



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> RESPONSE ELEMENT COMPOSITIONS AND ASSAYS EMPLOYING SAME  <b>(57) Abstract</b>  DNA segments have been discovered, and characterized by sequence, which are response elements operative to confer responsiveness to retinoic acid, or derivatives thereof, for the transcriptional activation of promoters in cells. By using transcriptional control regions comprising response elements of the present invention in combination with a functional promoter, it is now possible to provide recombinant DNA vectors containing a gene, the transcription (and, thereby, also expression) of which is under the control of a promoter, the transcriptional activity of which is responsive to (and increased by) retinoic acid or derivatives thereof.		

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RESPONSE ELEMENT COMPOSITIONS AND ASSAYS EMPLOYING SAME

This invention was made with Government support awarded by the United States National Institutes of Health. The United States Government has certain rights in the invention.

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FIELD OF INVENTION

The present invention relates to the  
10 superfamily of nuclear receptors known as the  
steroid/thyroid hormone receptors and their cognate  
response elements. More particularly, the present  
invention relates to the discovery of novel response  
elements which may be used to control the transcriptional  
15 activity of promoters.

BACKGROUND OF THE INVENTION

20 A central question in eukaryotic molecular  
biology is how specific DNA-binding proteins bind  
regulatory sequences to influence cell function and fate.  
The steroid/thyroid hormone receptors form a superfamily  
of ligand-dependent transcription factors that are  
25 believed to play a part in such cell function and fate.  
For example, it is known that these receptors transduce  
extracellular hormonal signals to target genes that  
contain specific enhancer sequences (referred to as  
hormone-response elements, or HREs). Each receptor  
30 contains a ligand-binding domain and a DNA-binding  
domain. The receptor undergoes a conformational change  
when it binds ligand. This conformational change permits  
the receptor-ligand complex to bind its cognate response  
element and thereby regulate transcriptional activity of

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an associated promoter. Transcriptional activation of promoter drives transcription of an operatively associated structural gene.

Sequence comparison and mutational analyses of hormone receptors, such as the glucocorticoid receptor (GR), have identified functional domains responsible for transcriptional activation and repression, nuclear localization, DNA binding, and hormone binding. The DNA binding domain, which is required in order to activate transcription, consists of 66-68 amino acids of which about 20 sites, including nine cysteines (C<sub>1</sub> to C<sub>9</sub>), are invariant among different receptors. The modular structure of members of this receptor superfamily allows the exchange of one domain for another to create functional, chimeric receptors.

The hormone response elements identified thus far are generally structurally related, but they are in fact functionally distinct. The response elements for GR [i.e., the glucocorticoid response element (GRE)], estrogen receptor [i.e., the estrogen response element (ERE)], and thyroid hormone receptor [i.e., the thyroid hormone response elements (TREs)] have been characterized in detail; they each consist of a palindromic pair of 'half sites' [Evans, *Science* 240, 889 (1988); Green and Chambon, *Trends in Genetics* 4, 309 (1988)]. With optimized pseudo- or consensus response elements, only two nucleotides per half site are different in GRE and ERE [Klock, et al., *Nature* 329, 734 (1987)]. On the other hand, identical half sites can be seen in ERE and TRE, but their spacing is different [Glass, et al., *Cell* 54, 313 (1988)]. Moreover, TRE has been shown to mediate transcriptional activation by transfected retinoic acid receptors (RARs) in CV-1 cells whereas non-transfected cells show no response. [Umesono et al., *Nature* 336, 262 (1988)]. In other words, both TR and RAR receptors can activate TREs.

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Since the response elements for only a few of the members of the steroid/thyroid superfamily have thus far been described, the response elements for other members of the superfamily remain to be described.

5

#### SUMMARY OF THE INVENTION

We have discovered, and characterized by sequence, DNA segments which are response elements operative to confer responsiveness to retinoic acid, or derivatives thereof, on the transcriptional activities of promoters in cells. We have also discovered that the transcriptional activity enhancing effect of the invention response elements occurs in all mammalian cells in the presence of retinoic acid or derivatives thereof, indicating that retinoic acid receptor, or retinoid X receptor, or other orphan receptors recognized by the invention response elements are present endogenously in all of these cells.

Contrary to what is reported in the art for the GRE, ERE and TRE, the novel response elements disclosed herein have a tandem repeat sequence, as opposed to a palindromic sequence reported for GRE, ERE and TRE. In addition, the invention response elements are much less susceptible to transcriptional activation by non-cognate receptors (e.g., estrogen receptor (ER), GR, thyroid hormone receptor (TR), etc.) than are the known response elements (GRE, ERE, TRE).

By using transcriptional control regions comprising response elements of the present invention and a functional promoter, it is now possible to provide recombinant DNA vectors containing a gene, the transcription (and, thereby, also expression) of which is under the control of a promoter, the transcriptional

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activity of which is responsive to (and increased by)  
retinoic acid or derivatives thereof.

5

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents a schematic diagram of a  
portion of the pCRBP11-CAT reporter plasmid.

10 Figure 2 summarizes transactivation of the  
response elements of the invention by RXR in the presence  
and absence of retinoic acid.

Figure 3a presents results of  
cotransfection of CV-1 cells with RAR or RXR expression  
plasmids and various reporter plasmids.

15 Figure 3b indicates the ability of RAR to  
block RXR's ability to transactivate pCRBP11-CAT in the  
presence (or absence) of retinoic acid. In the Figure,  
closed circles represent experiments run in the presence  
of retinoic acid, and open circles represent experiments  
20 run in the absence of retinoic acid.

#### DETAILED DESCRIPTION OF THE INVENTION

25 In accordance with the present invention,  
there is provided a substantially pure DNA having the  
sequence:

5'-NNNNNN-(N-NNNNNN)<sub>x</sub>-3',

wherein

30 each N is independently selected from A,  
T, C, or G; with the proviso that at least 3 nucleotides  
of each -NNNNNN- group of nucleotides are identical with  
the nucleotides at comparable positions of the sequence  
-AGGTCA-, and

x is a whole number of at least 1.

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Alternatively, the invention response elements can be described as substantially pure DNA having the sequence:

5'-AGGTCA-(N-AGGTCA)<sub>x</sub>-3',

5 wherein N and x are as defined above, and up to 3 nucleotides of each -AGGTCA- group of nucleotides can be replaced with N.

10 In accordance with another embodiment of the present invention, there are provided DNA constructs comprising the above-described response elements operatively linked to a promoter which is not normally subject to transcriptional activation by retinoic acid or derivatives thereof; wherein the DNA and the promoter are operatively linked so as to confer transcriptional  
15 activation activity on said promoter in the presence of a suitable ligand and its associated receptor. The above-described constructs can then be operatively linked to a gene for transcription. The resulting gene-containing DNA construct can then be incorporated into a vector for  
20 expression. The resulting vector can then be transformed into suitable host cells.

Cells containing the above-described vectors can then be used for the controlled expression of a gene of interest, in response to the presence or  
25 absence of a suitable ligand and its associated receptor.

In accordance with yet another embodiment of the present invention, there is provided a method for testing the activity of a test compound as an agonist of retinoic acid (or derivatives thereof), said method  
30 comprising:

(a) culturing a cell (as described above) in the presence of a retinoid receptor, and in the further presence, or in the absence, of the test compound; and thereafter

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(b) comparing the amount of the protein of interest expressed during the culturing in the presence, or in the absence, of the test compound.

5 In accordance with still another embodiment of the present invention, there is provided a method for testing the activity of a test compound as an antagonist of retinoic acid (or derivatives thereof), said method comprising:

10 (a) culturing a cell (as described above) in the presence of a retinoid receptor and retinoic acid (or derivatives thereof), and further:

(i) in the presence of the test compound, or  
(ii) in the absence of the test compound; and thereafter  
15

(b) comparing the amount of the protein of interest expressed during the (i) and (ii) culturing steps.

20 In accordance with a further embodiment of the present invention, there is provided a method to distinguish whether or not responsiveness to retinoic acid or derivatives thereof occurs via a pathway unique to RXR, relative to RAR, said method comprising:

25 contacting a vector containing an invention response element (as described above) with retinoic acid or derivatives thereof, and varying ratios of RAR:RXR expression vectors, and thereafter

determining the effect of increasing ratios of RAR:RXR on transcription activation of said response element by retinoic acid or derivatives thereof.  
30

35 In accordance with a still further embodiment of the present invention, there is provided a method to modulate RXR-induced expression in cells, said method comprising administering to said cells an amount of RAR effective to competitively bind, in the presence of retinoic acid or derivatives thereof, the response



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elements of the present invention, in preference to the binding thereof by RXR to the same DNA.

In accordance with another aspect of the present invention, there is provided a method to screen compounds to identify those compounds which act as ligands for orphan receptors, said method comprising:

contacting said compound with cells (as described above), wherein said cells are further transfected with an expression vector for an orphan receptor, wherein said orphan receptor, in the presence of its cognate ligand, is capable of binding to response elements of the present invention, and thereafter assaying for the expression of the reporter protein.

In the present specification and claims, reference is made to phrases and terms of art which are expressly defined for use herein as follows:

"RAR $\beta$ " or " $\beta$ RAR" both refer to retinoic acid receptor beta;

"CAT" means chloramphenicol acetyl transferase;

"LUC" means firefly luciferase;

" $\beta$ -Gal" means  $\beta$ -galactosidase;

"COS" means monkey kidney cells which express T antigen (Tag) [see, for example, Gluzman in Cell, 23: 175 (1981)];

"CV-1" means mouse kidney cells from the cell line referred to as "CV-1". CV-1 is the parental line of COS. Unlike COS cells, which have been transformed to express SV40 T antigen (Tag), CV-1 cells do not express T antigen;

"transcriptional control region" or "transcriptional control element" refer to a DNA segment comprising a response element operatively linked to a promoter to confer ligand responsiveness to transcriptional activity of the promoter;

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"operatively linked" means that the linkage (i.e., DNA segment) between the DNA segments so linked is such that the described effect of one of the linked segments on the other is capable of occurring. Effecting operable linkages for the various purposes stated herein is well within the skill of those of ordinary skill in the art, particularly with the teaching of the instant specification;

"promoter being naturally unresponsive to ligand" means that ligand does not enhance transcription from the promoter to an observable extent in a cell (e.g., a mammalian cell) unless a response element of the invention is spliced or inserted (upstream of the promoter) relative to the direction of transcription therefrom, by recombinant DNA or genetic engineering methods, into a DNA segment comprising the promoter, and linked to the promoter in a manner which makes transcriptional activity from the promoter operatively responsive to ligand;

"substantial sequence homology" refers to DNA or RNA sequences which have de minimus sequence variations from, and retain the same functions as, the actual sequences disclosed and claimed herein;

"retinoid receptors" refers to members of the steroid/thyroid superfamily of receptors which, in combination with retinoic acid, or derivatives thereof (as broadly defined herein), are effective to transcriptionally activate the response element(s) of the present invention, e.g., the retinoid X receptor [see, for example, Mangelsdorf, et al., in Nature 345: 224-229 (1990)], the ultraspiracle receptor [see, for example, Oro et al., in Nature 347: 298-301 (1990)], and the like;

"orphan receptors" refers to identified members of the steroid/thyroid superfamily of receptors for which specific ligands have not yet been identified. Examples include HNF4 [see, for example, Sladek et al.,

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in Genes & Development 4: 2353-2365 (1990)], the COUP family of receptors [see, for example, Miyajima et al., in Nucleic Acids Research 16: 11057-11074 (1988), Wang et al., in Nature 340: 163-166 (1989)], COUP-like receptors and COUP homologs, such as those described by Mlodzik et al., in Cell 60: 211-224 (1990) and Ladias et al., in Science 251: 561-565 (1991), the ultraspiracle receptor (see, for example, Oro et al., supra), and the like;

"suitable ligands" for hormone receptors refers to the specific ligand(s) which, in combination with its cognate receptor, is effective to transcriptionally activate the response element to which the cognate receptor binds;

"retinoic acid (RA) or derivatives thereof" refers broadly to retinoid compounds, including analogs and derivatives thereof, as described, for example, in The Retinoids, Volumes 1 and 2, Sporn, Roberts & Goodman, eds., Academic Press, Inc., New York (1984).

The nucleotides which occur in the various nucleotide sequences appearing herein have their usual single-letter designations (A, G, T, C or U) used routinely in the art.

In the present specification and claims, references to Greek letters may either be written out as alpha, beta, etc. or the corresponding Greek letter symbols (e.g.,  $\alpha$ ,  $\beta$ , etc.) may sometimes used.

The response elements of the present invention can be composed of two or more "half sites", wherein each half site comprises the sequence -NNNNNN-, with the proviso that at least 3 of the nucleotides in the half-site sequence are identical with the nucleotides at comparable positions of the sequence -AGGTCA-. Where one of the half sites varies by 3 nucleotides from the preferred sequence of -AGGTCA-, it is preferred that the other half site of the response element vary from the

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preferred sequence by less than 3 nucleotides. It is presently preferred that the 3'-half site (or downstream half site) of each pair of half sites vary from the preferred sequence by at most 1 nucleotide.

5 Exemplary response elements contemplated by the present invention are derived from various combinations of half sites having sequences selected from, for example, -AGGTCA-, -AGTTCA-, -CTGTCA-,  
-GGGTCA-, -ATTTCA-, -ATGTCA-, -CGGTCA-, -AGCTCA-,  
10 -AGGCCA-, -AGGTGA-, -AGGTTA-, -AGGTCC-, -ATGTGC-, and the like. Presently preferred half sites are those having sequences selected from -AGGTCA-, -AGTTCA-, or -CTGTCA-.

While the spacer nucleotide can be any one of C, T, G, or A, it is presently preferred to use C as  
15 the spacer nucleotide, because this is the spacer most commonly observed in response elements found in nature.

Presently preferred response elements contemplated by the present invention include:

5'-AGGTCA-C-AGGTCA-3',  
20 5'-AGGTCA-C-AGGTCA-C-AGGTCA-C-AGTTCA-3',  
5'-CTGTCA-C-AGGTCA-C-AGGTCA-C-AGGTCA-C-AGTTCA-  
3', and the like.

With respect to the promoter which is part of a transcriptional control region of the invention,  
25 practically any promoter may be used, so long as the transcriptional activity of such a promoter can be enhanced by a response element of the present invention (when suitably positioned upstream from the promoter),  
provided that such promoter is naturally unresponsive, in  
30 its transcriptional activity, to retinoic acid or derivatives thereof. Among such promoters are Delta-MTV promoter of mouse mammary tumor virus, Herpes simplex thymidine kinase (tk) promoter, basal Simian virus SV-40 promoter, the Drosophila alcohol dehydrogenase (ADH)  
35 promoter, and the like. Presently preferred are promoters which require a response element for activity.

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Virtually any protein or polypeptide of interest can be made with cells transformed with an expression vector of the invention. Such proteins include hormones, lymphokines, receptors or receptor subunits, immunoglobulin chains and the like. Indicator proteins such as LUC, CAT, and  $\beta$ -Gal can also be made.

Among the types of cells that can be transformed in accordance with the invention are mammalian cells, avian cells, insect cells, and the like, such as, for example, CV-1, COS, F9, P19, CHO, HeLa, NIH 3T3, HuTu80, Rat2 fibroblasts, HT1080.T, chick embryo fibroblasts, quail QT6, Drosophila Schneider S2 cells, and the like.

The invention method for determining the activity of a test compound as an agonist or antagonist of retinoic acid or derivatives thereof can be carried out employing standard assay techniques, as are well known by those of skill in the art. See, for example, Mangelsdorf et al., in Nature 345: 224-229 (1990).

Test compounds contemplated for screening in accordance with the invention assay methods include any compound which can potentially affect the ability of receptor to promote trans-activation through a response element of the present invention.

In accordance with a specific embodiment of the present invention, wherein it is possible to distinguish whether responsiveness to retinoic acid (or derivatives thereof) occurs via a pathway unique to RXR (relative to RAR) or via some other pathway, responsiveness to retinoic acid (or derivatives thereof) via the RAR pathway would result in increased amounts of transactivation as a function of increasing RAR expression, while responsiveness to retinoic acid (or derivatives thereof) via the RXR pathway would result in reduced levels of transactivation as a function of increased RAR expression (reduced levels of

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transactivation would be caused by RAR progressively blocking activation by RXR).

Receptors, assay methods, and other subject matter pertinent to the subject matter of the present specification may be found in the following references, which are incorporated herein by reference: Commonly assigned United States Patent Application Serial No. 108,471, filed October 20, 1987 and published as PCT International Publication No. WO 88,03168; commonly assigned United States Patent Application Serial No. 276,536, filed November 30, 1988 and published as European Patent Application Publication No. 0 325 849; commonly assigned United States Patent Application Serial No. 370,407, filed June 22, 1989, said Application listing a Budapest Treaty Deposit of a plasmid harboring a cDNA encoding a gamma-retinoic acid receptor, said deposit having been made June 22, 1989, and bearing American Type Culture Collection Accession No. 40623; Zelent et al., Nature 339, 714 (1989); Petkovich et al., Nature 330, 444 (1987); Brand et al., Nature 332, 850 (1988).

Because the DNA segments which comprise the response elements of the present invention are relatively short, they may be provided synthetically, that is by synthesizing the response element-containing oligonucleotide on a DNA synthesizer as is known in the art. It is frequently very desirable to provide restriction endonuclease sites at the 3'- and 5'-ends of the oligomer, such that the synthetic response element may be conveniently inserted into a DNA expression vector at a site upstream from the promoter, whose transcriptional activity is to be enhanced and which drives transcription of the desired gene. As those of ordinary skill in the art will understand, the response elements of the present invention, like other response elements, are orientation and, with wide latitude,

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position independent. Thus, the response elements of the present invention are functional in either orientation and may be placed in any convenient location from about 30 nucleotides upstream to about 10,000 nucleotides upstream from the promoter to be affected.

Preferred cells for use with expression systems employing transcriptional control regions comprising invention response element are mammalian cells such as COS cells and CV-1 cells. COS-1 (referred to as COS) cells are mouse kidney cells that express SV40 T antigen (Tag); while CV-1 cells do not express SV40 Tag. CV-1 cells are convenient because they lack any endogenous glucocorticoid or mineralocorticoid or other known steroid or thyroid hormone receptors, except that they do produce low levels of  $\beta$ RAR. Thus, via gene transfer with appropriate expression vectors comprising a heterologous gene under the control of a transcriptional control region of the invention, it is possible to convert these host cells into transformed cells which produce increased quantities of a desired protein in response to induction by retinoic acid or derivatives thereof.

Expression plasmids containing the SV40 origin of replication can propagate to high copy number in any host cell which expresses SV40 Tag. Thus, expression plasmids carrying the SV40 origin of replication can replicate in COS cells, but not in CV-1 cells. Although increased expression afforded by high copy number is desirable, it is not critical to the assay systems described herein. The use of any particular cell line as a host is also not critical, although CV-1 cells are presently preferred because they are particularly convenient.

The invention will now be described in detail by reference to the following non-limiting example.

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EXAMPLE

Cellular retinol binding protein type II (CRBP<sub>II</sub>) is an abundant intestinal protein. A portion of the rat CRBP<sub>II</sub> gene promoter, from position -988 to +50 [see Demmer, et al., in J. Biol. Chem. 262: 2425-2467 (1987)], was introduced into a plasmid which would regulate expression of the reporter, bacterial chloramphenicol acetyl transferase (see Fig. 1). The resultant reporter plasmid, pCRBP<sub>II</sub>-CAT, was tested for retinoic acid (RA) responsiveness after transfection into mouse embryonal teratocarcinoma F9 cells. F9 cells endogenously express high amounts of retinoic acid receptor (RAR), and can efficiently transactivate (>100-fold) a reporter plasmid containing the well characterized RA-inducible promoter of the RAR $\beta$  gene [see de The et al., in Nature 343: 177-180 (1990) and Sucov, et al., in Proc. Natl. Acad. Sci. U.S.A. 87: 5392-5396 (1990)]. However, pCRBP<sub>II</sub>-CAT conferred no basal or RA-inducible CAT activity in these cells. To eliminate the possibility that regulation of the CRBP<sub>II</sub> promoter requires an augmented concentration of RAR, as is the case for the laminin retinoic acid response element [RARE; see Vasias, et al., in Proc. Natl Acad. Sci. U.S.A. 86: 9099-9103 (1989)], or that its regulation is isoform specific, expression vectors bearing all three subtypes of RAR were each separately cotransfected with pCRBP<sub>II</sub>-CAT into F9 cells.

Even after overexpression, RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$  are not able to confer a RA response on pCRBP<sub>II</sub>-CAT. It was next tested whether this were also true for the retinoid X receptor (RXR). The RXRs are members of the nuclear receptor superfamily which also respond specifically to RA but which are substantially different in primary structure and ligand specificity from the RARs [see Mangelsdorf, et al., in Nature 345: 224-229 (1990)].



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Remarkably, RA provoked a dramatic increase (100-fold) in CAT activity when the expression vector encoding the human RXR $\alpha$  was cotransfected with the pCRBP<sub>II</sub>-CAT reporter into F9 cells. This induction of the CRBP<sub>II</sub> promoter by RXR is dependent on both the concentration of ligand and the concentration of receptor.

To assess the exact sequences within the CRBP<sub>II</sub> promoter that function as an RXR response element (RXRE), a series of 5'-deletion mutants were constructed starting with the parent reporter plasmid pCRBP<sub>II</sub>-CAT (see Fig. 2). In the absence of RA, all of these constructs exhibited very low CAT activity in F9 cells cotransfected with RXR. After addition of RA, full RXR-dependent transactivation could still be seen when promoter sequences were deleted to -741 (pCRBP<sub>II</sub> $_{\Delta 5}$ -CAT). However, further deletions to -604 (pCRBP<sub>II</sub> $_{\Delta H}$ -CAT) and -313 (pCRBP<sub>II</sub> $_{\Delta P}$ -CAT) abolished this response. These results indicate that an RXRE is located between bases -741 to -604 in the CRBP<sub>II</sub> gene promoter. Inspection of this region reveals a striking sequence of bases from -639 to -604 which contains five nearly perfect tandem repeats of -AGGTCAC-. Deletion of the sequence including these half-sites (p $\Delta$ CRBP<sub>II</sub>-CAT, Fig. 2) eliminated RXR-dependent induction, providing direct evidence that this sequence functions as an RXRE. Significantly, the CRBP<sub>II</sub>-RXRE can be fused to an heterologous promoter, such as the minimal SV40 early promoter, and confer RA and RXR dependent activation (see p $\Delta$ SV-CRBP<sub>II</sub>-CAT in Fig. 2; see also below).

To demonstrate that the CRBP<sub>II</sub>-RXRE is an authentic binding site for RXR, partially purified, bacterially synthesized receptor proteins were examined by gel retardation assays using [<sup>32</sup>P]-labeled DNA probes. As a control, RAR was also included in these studies. Previous work has shown that bacterially produced RAR is competent for both ligand and DNA binding [see Yang, et

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al., in Proc. Natl. Acad. Sci. U.S.A. 88: in press (1991)]. Surprisingly, when increasing amounts of RAR were incubated with [<sup>32</sup>P] CRBP<sub>II</sub>-RXRE, a major protein-DNA complex could be formed which eventually saturated all of the labeled probe. The binding affinity of RAR to the RXRE appeared to be even greater than its binding to  $\beta$ RE (a known RARE; see de The, et al., Supra, and Sucov, et al., Supra). In addition, unlabeled CRBP<sub>II</sub>-RXRE competed at least as well as unlabeled  $\beta$ RE for [<sup>32</sup>P]  $\beta$ RE binding to RAR. The specificity of the RAR for both  $\beta$ RE and CRBP<sub>II</sub>-RXRE are further demonstrated by the inability of a nonspecific oligonucleotide (GRE<sub>r</sub>) to compete.

When a similar analysis was performed using RXR proteins, another unexpected result was observed. While increasing amounts of RXR bound the CRBP<sub>II</sub>-RXRE probe strongly and specifically, as expected, the protein-DNA complex which is formed between RXR and the RXRE migrates with a much slower electrophoretic mobility than does the complex formed between RAR and the RXRE (even though both receptors are the same size). Further, the  $\beta$ RE, which is a weak RXR response element and does not compete well with CRBP<sub>II</sub>-RXRE for RXR binding, also forms a much weaker and faster migrating complex with RXR than does the CRBP<sub>II</sub>-RXRE. These results indicate that RXR binds to the CRBP<sub>II</sub>-RXRE as a multimer, whereas the RAR does not. This higher order complex may contribute to the RXR's exclusive ability to transactivate the CRBP<sub>II</sub> gene promoter. Within the CRBP<sub>II</sub>-RXRE there are four perfect direct repeats of the canonical half-element -AGGTCA-, each separated by one nucleotide. This suggests that the RXR binds to CRBP<sub>II</sub>-RXRE as a tetramer.

Since RAR tightly binds the CRBP<sub>II</sub>-RXRE, yet does not transactivate the CRBP<sub>II</sub> gene promoter, the potential role of RAR in regulating CRBP<sub>II</sub> expression was investigated. To investigate the specificity of the

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retinoid receptors for the RXRE, cotransfection studies were performed in CV-1 cells, which, unlike F9 cells, do not express high endogenous levels of RAR. The reporter plasmids used in these experiments were constructed by introducing TRE<sub>p</sub>, a known RARE and RXRE [see Mangelsdorf, et al., in Nature 345: 224-229 (1990) and Umesono, et al., in Nature 336: 262-265 (1988)], and CRBP<sub>II</sub>-RXRE into the minimal promoter of pΔSV-CAT (K. Umesono and R. Evans, in preparation). As shown in Fig. 3 and as previously demonstrated (Umesono et al., Supra), TRE<sub>p</sub> responds markedly to RA in the presence of either RAR or RXR. However, in agreement with experiments on the native CRBP<sub>II</sub> promoter (Fig. 2), the RXRE is RA-responsive only in the presence of RXR.

It was next tested to see whether the ability of the RAR to bind the RXRE would also enable it to block RXR transactivation. When increasing amounts of RAR expression plasmids were cotransfected with a fixed concentration of RXR expression plasmid, RXR activation decreased dramatically and was 90% suppressed when the concentration of expression vectors for RAR and RXR was equal (see Fig. 3b). These results suggest that vitamin A metabolites are able to feedback regulate the expression of CRBP<sub>II</sub> and that this regulation can be either positive or negative depending on the type of retinoid receptor present and its concentration. The discovery of the CRBP<sub>II</sub>-RXRE assigns a specific role to the RXR in modulating vitamin A homeostasis and provides a useful tool for distinguishing between the pathways of RXR and RAR action.

While the invention has been described in detail with reference to preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed herein.

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CLAIMS

THAT WHICH IS CLAIMED IS:

- 5                   1. A substantially pure DNA having the sequence:

5'-NNNNNN-(N-NNNNNN)<sub>x</sub>-3',

wherein

- 10                   each N is independently selected from A, T, C, or G; with the proviso that at least 3 nucleotides of each -NNNNNN- group of nucleotides are identical with the nucleotides at comparable positions of the sequence -AGGTCA-, and
- x is a whole number of at least 1.

15

2. A DNA according to Claim 1 wherein x is 1 and each -NNNNNN- group of nucleotides have the sequence -AGGTCA-.

20

3. A DNA according to Claim 2 wherein -N- is -C-.

25

4. A DNA according to Claim 1 wherein x is 3 and each -NNNNNN- group of nucleotides have the sequence -AGGTCA- or -AGTTCA-.

30

5. A DNA according to Claim 4 wherein -N- is -C-.

6. A DNA according to Claim 5 having the sequence:

5'-AGGTCA-C-AGGTCA-C-AGGTCA-C-AGTTCA-3'.

35

7. A DNA according to Claim 1 wherein x is 4 and each -NNNNNN- group of nucleotides have the sequence -AGGTCA-, -AGTTCA-, or -CTGTCA-.

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8. A DNA according to Claim 7 wherein -N- is  
-C-.

5       9. A DNA according to Claim 8 having the  
sequence:  
5'-CTGTCA-C-AGGTCA-C-AGGTCA-C-AGGTCA-C-AGTTCA-  
3'.

10       10. A substantially pure DNA construct  
comprising the DNA of Claim 1 operatively linked to a  
promoter which is not normally subject to transcriptional  
activation by retinoic acid or derivatives thereof;  
wherein the DNA and the promoter are operatively linked  
so as to confer transcriptional activation activity on  
15       said promoter in the presence of a suitable ligand and  
its associated receptor.

20       11. A DNA construct according to Claim 10  
wherein said suitable ligand and its associated receptor  
comprise retinoic acid or derivatives thereof, and RXR.

25       12. A DNA construct according to Claim 10  
wherein the promoter is selected from the delta-MTV  
promoter of mouse mammary tumor virus, the SV40 early  
promoter, the Herpes simplex virus thymidine kinase  
promoter, or the Drosophila alcohol dehydrogenase  
promoter.

30       13. A substantially pure DNA construct  
comprising the DNA construct of Claim 10 linked  
operatively for transcription to a gene.

35       14. A vector for the expression of a protein  
of interest in a cell, said vector comprising the  
substantially pure DNA construct of Claim 13, wherein  
said gene encodes the protein of interest.

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15. A vector according to Claim 14 wherein the protein of interest is selected from luciferase, chloramphenicol acetyl transferase, or  $\beta$ -galactosidase.

5           16. A cell transformed with the vector of Claim 14.

10           17. A cell according to Claim 16 wherein the cell is a mammalian, avian or insect cell of a type selected from CV-1, COS, F9, CHO, HeLa, NIH 3T3, HuTu80, Rat2 fibroblasts, HT1080.T, chick embryo fibroblasts, quail QT6, or Drosophila Schneider S2 cells.

15           18. A method for the controlled expression of a gene of interest, said method comprising culturing cells of Claim 16 in the presence or absence of a suitable ligand and its associated receptor.

20           19. A method according to Claim 18 wherein said suitable ligand and its associated receptor comprise retinoic acid or derivatives thereof, and RXR.

25           20. A method for testing the activity of a test compound as an agonist of retinoic acid or derivatives thereof, said method comprising:

(a) culturing a cell according to Claim 16 in the presence of retinoid receptor, and in the further presence, or in the absence, of the test compound; and thereafter

30           (b) comparing the amount of said protein expressed during the culturing in the presence, or in the absence, of the test compound.

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21. A method for testing the activity of a test compound as an antagonist of retinoic acid or derivatives thereof, said method comprising:

5 (a) culturing a cell according to Claim 16 in the presence of a retinoid receptor and retinoic acid or derivatives thereof, and further:

(i) in the presence of the test compound,  
or

10 (ii) in the absence of the test compound;  
and thereafter

(b) comparing the amount of said protein expressed during the (i) and (ii) culturing steps.

15 22. A method to distinguish whether or not responsiveness to retinoic acid or derivatives thereof occurs via a pathway unique to RXR, relative to RAR, said method comprising:

20 contacting a vector according to Claim 14 with retinoic acid or derivatives thereof, and varying ratios of RAR:RXR expression vectors, and thereafter

determining the effect of increasing ratios of RAR:RXR on transcription activation of said response element by retinoic acid or derivatives thereof.

25 23. A method to modulate RXR-induced expression in cells, said method comprising administering to said cells an amount of RAR effective to competitively bind the DNA of Claim 1, in the presence of retinoic acid or derivatives thereof, in preference to binding by RXR  
30 to the same DNA.

24. A method to screen compounds to identify those compounds which act as ligands for orphan receptors, said method comprising:

35 contacting said compound with cells according to Claim 16, wherein said cells are further transfected

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with an expression vector for an orphan receptor, wherein said orphan receptor, in the presence of its cognate ligand, is capable of binding to a substantially pure DNA having the sequence:

5                                   5'-NNNNNN-(N-NNNNNN)<sub>x</sub>-3',

wherein

each N is independently selected from A, T, C, or G; with the proviso that at least 3 nucleotides of each -NNNNNN- group of nucleotides are identical with the nucleotides at comparable positions of the sequence -AGGTCA-, and

x is a whole number of at least 1, and thereafter

assaying for the expression of said protein of interest.

15



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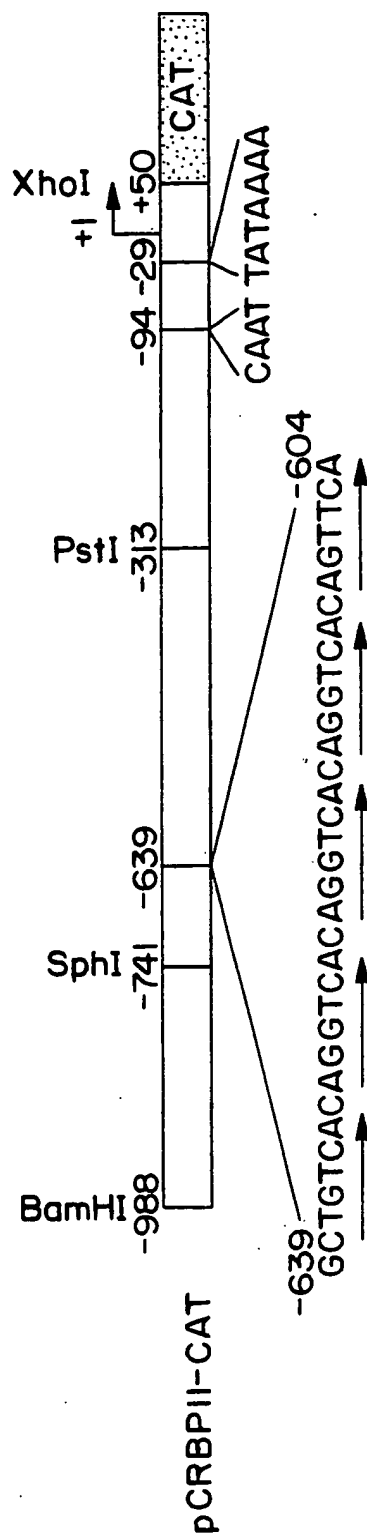


FIG. 1

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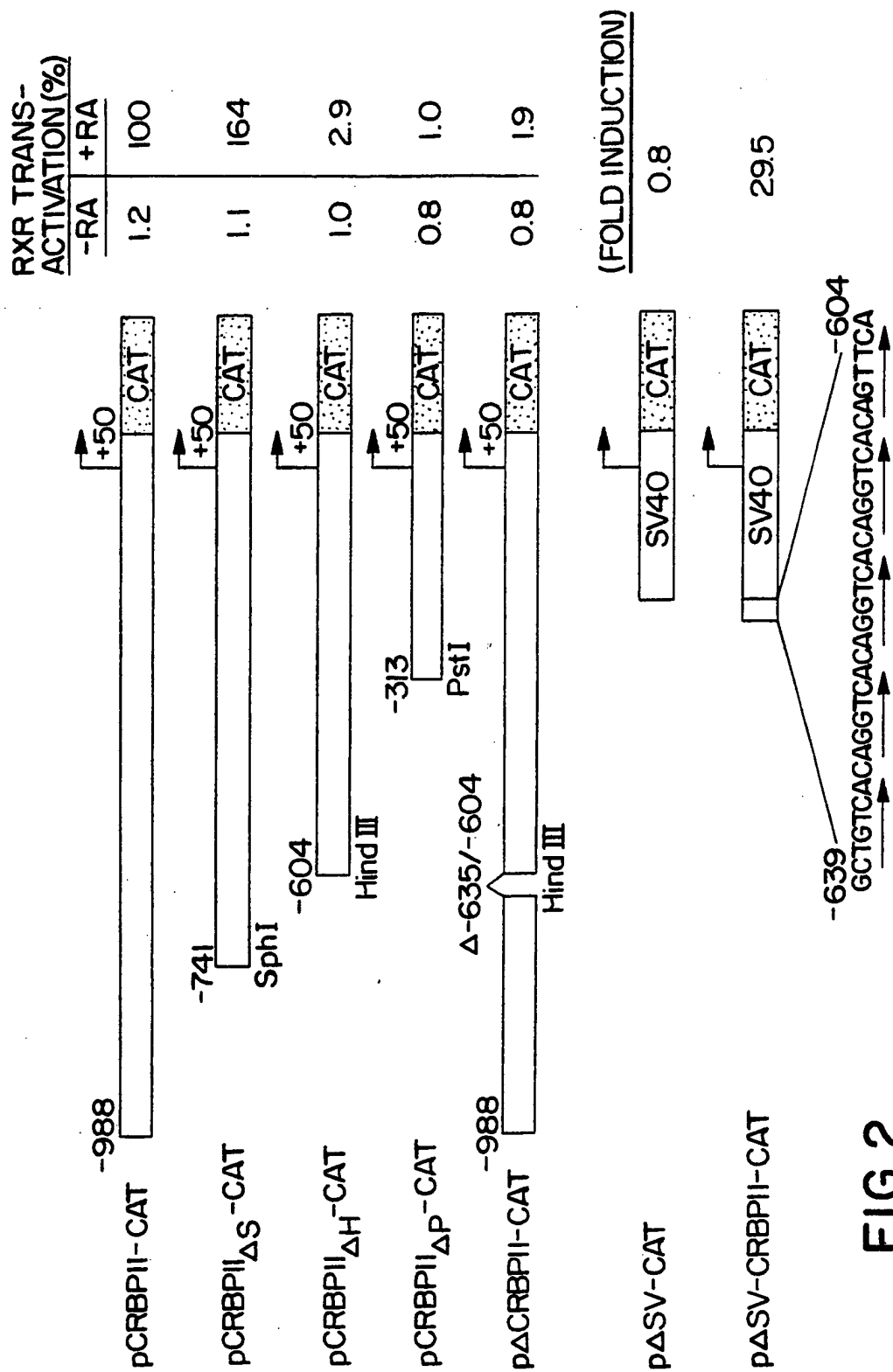


FIG. 2

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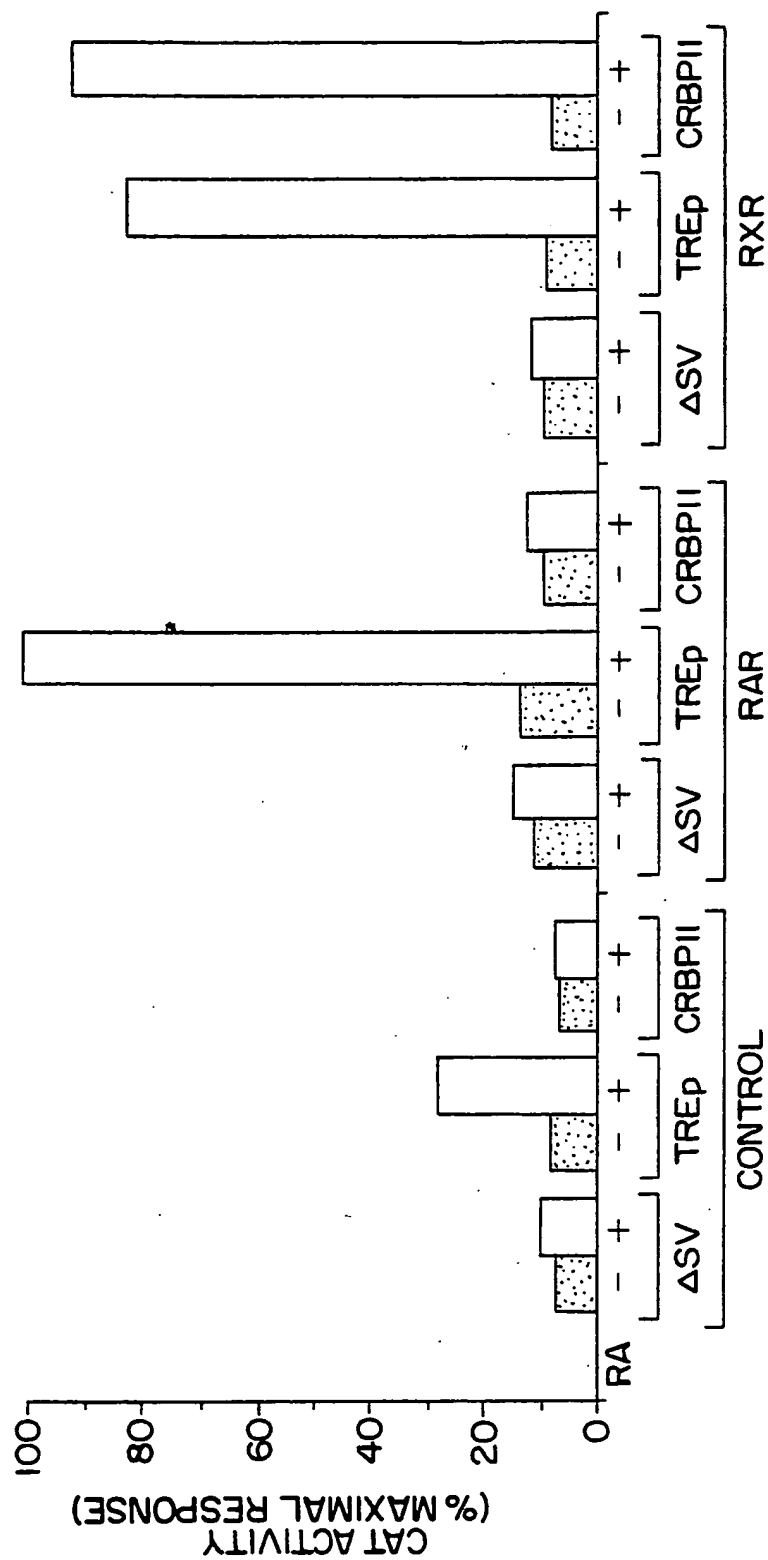


FIG. 3a

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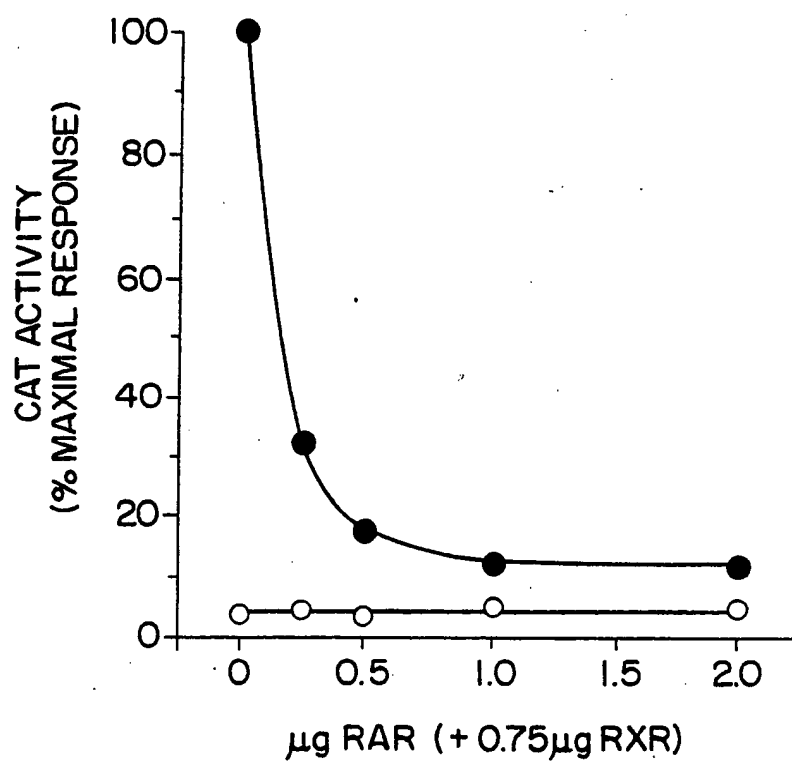


FIG. 3b

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02014

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5) : C12Q 1/68 US CL : 435/6		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	435/6	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
APS, Dialog, Intelligenetics		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
A	Science, Vol. 240, issued 13 May 1988, R.M. Evans, "The steroid and thyroid hormone receptor superfamily", pages 889-895, see entire document.	1-24
X/Y	Cell, Vol. 55, issued 21 October 1988, S.Y. Tsai et al., "Molecular interactions of steroid hormone receptor with its enhancer element: Evidence for receptor dimer formation", pages 361-369, see entire document.	1-18/19-24
X/Y	Cell, Vol. 57, issued 30 June 1989, K. Umesono and R.M. Evans, "Determinants of target gene specificity for steroid/thyroid hormone receptors", pages 1139-1146, see entire document.	1-18/19-24
X/Y,P	Cell, Vol. 65, issued 28 June 1991, K. Umesono et al., "Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors", pages 1255-1266, see entire document.	1-18/19-24
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:<sup>16</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>	Date of Mailing of this International Search Report <sup>2</sup>	
26 June 1992	30 JUN 1992	
International Searching Authority <sup>1</sup>	Signature of Authorized Officer <sup>20</sup>	
ISA/US	KAREN COCHRANE CARLSON, PH.D.	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
X/Y,P	Cell, Vol. 66, issued 09 August 1991, D.J. Mangelsdorf et al., "A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR", pages 555-561, see entire document.	1-19/20-24
X/Y,P	Proc. Natl. Acad. Sci., USA, Vol. 89, issued February 1992, S.A. Kliewer et al., "Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling", pages 1448-1452, see entire document.	1-19/20-24
X,P	US, A, 5,091,518 (H.M. Sucov et al) 25 February 1992, see entire document	1-24

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

1. ☐ Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category <sup>16</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
X, E	Biochem. Biophys. Res. Commun., Vol. 183, No. 2, issued 16 March 1992, Y. Kadowaki et al., "EAR3-COUP-TF binds most tightly to a response element with tandem repeat separated by one nucleotide", pages 492-498, see entire document.	1-24

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